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Field Evaluation of Dried Blood Spots for Routine HIV-1 Viral Load and Drug Resistance Monitoring in Patients Receiving Antiretroviral Therapy in Africa and Asia

Marjorie Monleau,[a]* Avelin F. Aghokeng,[a,b] Sabrina Eymard-Duvernay,[a] Anoumou Dagnra,[c] Dramane Kania,[d] Nicole Ngo-Giang-Huong,[e] Coumba Touré-Kane,[f] Lien X. T. Truong,[g] Marie-Laure Chaix,[h] Eric Delaporte,[a] Ahidjo Ayouba,[a] Martine Peeters,[a] for the ANRS 12235 Study Group

UMI 233 TransVIHMI, IRD, and Université de Montpellier 1, Montpellier, France[a]; Virology Laboratory, CREMER/IMPM/IRD, Yaoundé, Cameroon[b]; Centre National de Référence VIH-IST/PNLS, BIOLIM, FMMP/UL, Lomé, Togo[c]; Laboratoire de Virologie, Centre Muraz, Bobo-Dioulasso, Burkina Faso[d]; IRD UMI 174-PHPT and Chiang Mai University, Chiang Mai, Thailand[e]; Laboratoire de Bactériologie-Virologie, Dakar, Senegal[f]; Laboratoire de VIH/SIDA, Institut Pasteur, Ho Chi Minh Ville, Vietnam[g]; Université Paris Descartes, EA 3620, PRES Sorbonne Paris Cité, AP-HP, Laboratoire de Virologie, CHU Necker-Enfants Malades, Paris, France[h]

Dried blood spots (DBS) can be used in developing countries to alleviate the logistic constraints of using blood plasma specimens for viral load (VL) and HIV drug resistance (HIVDR) testing, but they should be assessed under field conditions. Between 2009 and 2011, we collected paired plasma-DBS samples from treatment-experienced HIV-1-infected adults in Burkina Faso, Cameroon, Senegal, Togo, Thailand, and Vietnam. The DBS were stored at an ambient temperature for 2 to 4 weeks and subsequently at −20°C before testing. VL testing was performed on the plasma samples and DBS using locally available methods: the Abbott m2000rt HIV-1 test, generic G2 real-time PCR, or the NucliSENS EasyQ version 1.2 test. In the case of virological failure (VF), i.e., a plasma VL of ≥1,000 copies/ml, HIVDR genotyping was performed on paired plasma-DBS samples. Overall, we compared 382 plasma-DBS sample pairs for DBS VL testing accuracy. The sensitivities of the different assays in different laboratories for detecting VF using DBS varied from 75% to 100% for the m2000rt test in labs B, C, and D, 91% to 93% for generic G2 real-time PCR in labs A and F, and 85% for the NucliSENS test in lab E. The specificities varied from 82% to 97% for the m2000rt and NucliSENS tests and reached only 60% for the generic G2 test. The NucliSENS test showed good agreement between plasma and DBS VL but underestimated the DBS VL. The lowest agreement was observed for the generic G2 test. Genotyping was successful for 96/124 (77%) DBS tested, and 75/96 (78%) plasma-DBS pairs had identical HIVDR mutations. Significant discrepancies in resistance interpretations were observed in 9 cases, 6 of which were from the same laboratory. DBS can be successfully used as an alternative to blood plasma samples for routine VL and HIVDR monitoring in African and Asian settings. However, the selection of an adequate VL measurement method and the definition of the VF threshold should be considered, and laboratory performance should be monitored.

The increasing availability of antiretroviral treatment (ART) has dramatically contributed to a reduction in mortality and morbidity related to HIV/AIDS in resource-limited countries (RLC). In order to limit the emergence of resistance to antiretroviral drugs, HIV treatment should ideally be accompanied by regular virological monitoring, including viral load (VL) and genotypic drug resistance testing (1). With the recent scaling up of ART in RLC, people from semiurban and rural areas are now also receiving treatment, and recent data have shown variable levels of virological failure (VF) and drug resistance in these areas. In certain settings, VF can be >20% after 12 months of ART (2–4), stressing the urgent need for improved virological monitoring.

In the majority of developing countries with limited resources, monitoring blood plasma VL poses logistical challenges since plasma preparation, storage, and/or shipment requires personnel and laboratory infrastructure that is often lacking. Today, viral load assays still require sophisticated equipment and specialized personnel and are therefore essentially implemented in reference laboratories in major cities. Dried blood spots (DBS) offer the advantages of a stable specimen matrix, ease of sample collection, and shipment with minimal biohazard risks (5, 6). Several studies have recently indicated that HIV-1 VL and genotypic drug resistance testing using DBS are feasible and have comparable performances to those done with plasma samples (7–10). DBS can be stored at ambient temperature for 2 to 4 weeks in hermetic bags with a desiccant and can be subsequently kept for long periods when refrigerated or frozen until the time of genotypic drug resistance testing or RNA quantification (6, 11). However, certain limitations and challenges remain with their operational use, the major one being the lower limit of quantification than that of plasma and/or the interference of intracellular DNA and RNA, which can depend on the methods used for nucleic acid extraction or the assay principle (nucleic acid sequence-based amplification [NASBA], real-time PCR, or others) but also on the overall experience of the laboratory with the use of DBS.

Recently, several RLC programs have started to use DBS to

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Address correspondence to Avelin F. Aghokeng, avelin.aghokeng@ird.fr.
* Present address: Marjorie Monleau, CNRS UMR 5535, Institut de Genetique Moleculaire de Montpellier, Montpellier, France.

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expand access to HIV VL and genotypic drug resistance testing (12–14). It is thus important to evaluate to what extent this approach is reliable compared to using plasma, also considering the existing infrastructure and personnel. In this study, we compared VL measurements and drug resistance mutations (DRMs) obtained in parallel on blood plasma and DBS samples in six different countries with different laboratory platforms and programs.

MATERIALS AND METHODS

Study sites and population. Between October 2009 and December 2011, we implemented a cross-sectional evaluation of treatment outcome in clinics that administered antiretrovirals (ARVs) and monitored this treatment according to national recommendations in Burkina Faso, Cameroon, Senegal, Togo, Thailand, and Vietnam. Consenting study participants were consecutively enrolled and were HIV-1-infected adults (≥18 years old) attending health care services for their routine medical visits and who had 24 ± 2 months ART experience at a unique treatment site. All were still taking their first-line treatment regimen and reported no history of previous exposure to ARVs prior to treatment initiation (2).

Sample collection and preparation. Whole-blood samples were collected from 1,618 eligible patients and directly used in recruitment sites to prepare DBS before shipment of remaining samples to designated national laboratories. Upon receipt, the remaining blood samples were centrifuged and the blood plasma specimens aliquoted and stored at −70°C for further HIV-1 RNA quantification and drug resistance genotyping. For DBS preparation, 50 μl of whole blood was spotted on each of five circles of a Whatman 903 filter paper and dried at ambient temperature for 3 h. Two DBS cards were prepared for each patient, placed in individual ziplock plastic bags containing two silica desiccants, and stored in a hermetic box also containing silica desiccants. The DBS samples were initially stored at an ambient temperature for between 2 and 4 weeks and were subsequently shipped to designated laboratories for storage at −20°C for further HIV-1 RNA quantification and drug resistance genotyping. The desiccants were checked at regular time intervals for the presence of humidity and were replaced when necessary.

RNA isolation and viral load determination. Viral load was determined with the methods routinely used in each laboratory. Overall, two different detection principles corresponding to three commercial methods were used: nucleic acid sequence-based amplification (NASBA) real-time detection corresponding to the NucliSENS EasyQ HIV-1 version 1.2 assay (bioMérieux, Marcy l'Etoile, France) and the real-time PCR detection method corresponding to the m2000rt RealTime HIV-1 assay (Abbott, Abbott Park, IL, USA) and the generic G2 real-time PCR assays (Biocentric, Bandol, France). We randomly encoded the participating countries and laboratories as lab A, lab B, lab C, lab D, lab E, and lab F. The NucliSENS technique was used in lab E, the m2000rt technique was used in labs B, C, and D, and the generic G2 method was used in labs A and F. For VL quantification with DBS using the generic G2 method, the Qiagen extraction kit (Qiagen, Courtaboeuf, France), which is recommended by the manufacturer for plasma samples, was not used because of its known low performance for RNA extraction from DBS (9), and it was replaced in both labs by the NucliSENS miniMag extraction system. For all these methods, VL measurements on the plasma specimens were performed according to the manufacturer's recommendations. For DBS testing, an initial stage was required to recover nucleic acids from the filter papers. Briefly, 2 entire spots were incubated in 1.7 ml (m2000rt) or in 2 ml (NucliSENS miniMag) of lysis buffer for 30 min under constant shaking. The recovered eluate was transferred to 2-ml collection tubes and centrifuged for 10 min at 2,000 rpm to eliminate paper particles from the supernatant. The supernatants were used similarly to the plasma specimens for RNA extraction and VL determination. At each site, approximately 60 DBS were tested for VL. This number included all samples with a plasma VL of ≥1,000 copies/ml and about 10% of samples with a plasma VL between the assay quantification threshold and 1,000 copies/ml, 10% with a plasma VL below the assay quantification limit, and 10% with an undetectable plasma VL.

Genotypic drug resistance testing. All blood plasma samples with a VL of ≥1,000 copies/ml were genotyped to investigate viral resistance to treatment. DBS were selected for drug resistance genotyping if plasma RNA was ≥1,000 copies/ml and the DBS VL was ≥5,000 copies/ml. The genotyping assays were performed locally at labs B, C, D, and F. For labs A and E, drug resistance testing was conducted in collaborating reference laboratories, the Necker Hospital virology laboratory (Paris, France) and the IRD UMI-233 TransVIHMI laboratory (Montpellier, France), respectively. Genotyping on plasma specimens was performed with the ViroSeq HIV-1 system (Celera Diagnostics, Alameda, CA) (15) at lab B, while labs C, D and F and the two laboratories in France used the Agence Nationale de Recherche sur le Sida (ANRS) in-house protocol (see www.hivfrenchresistance.org/ANRS-procedures.pdf). Both the ViroSeq and the in-house methods cover the entire protease and at least the first 240 codons of the reverse transcriptase (RT).

For genotyping on DBS, nucleic acids were extracted using either the m2000rt method or the NucliSENS miniMag method as described above for VL determination. All laboratories amplified the protease and RT regions separately to optimize the PCR results. This was achieved in labs C, D, and F and the IRD UMI-233 lab in France using the ANRS in-house protocol that amplifies protease and RT fragments separately, yielding fragments of 507 bp and 798 bp, respectively (www.hivfrenchresistance.org/ANRS-procedures.pdf). A similar approach was used in lab B but with different primer sets: a one-step RT-PCR was performed with forward primer PR2 (HXB2 positions 1557 to 1583, 5′-CCTAGRAAAARG GGCTGTTGGAAATGT-3′) and reverse primer TR2as (HXB2 positions 2915 to 2941, 5′-AATYTGACTTGCCCARTTTARTTTTCC-3′). Separated nested PCRs were performed in the protease (amino acids 1 to 99) using PR3 (HXB2 positions 1590 to 1615, 5′-GARGGACAYCAAATGAA AGAYTGYAC-3′) and PR3as (HXB2 positions 2176 to 2204, 5′-GCCAT TGTTTAACYTTTGGDCCATCCATT-3′) and in the RT (amino acids 1 to 260) with TR3 (HXB2 positions 1971 to 1998, 5′-TGATAGGRGGAA TTGGAGGTTTTATCAA-3′) and TR3as (HXB2 positions 2885 to 2912, 5′-CTAAYTTYTGTATRTCATTGACAGTCCA-3′). Each reverse transcription-PCR was performed with 10 μl of RNA using the SuperScript one-step RT-PCR method for long templates (Invitrogen Life Technologies, Saint Aubin, France). Next, 5 μl of the first-round amplification product was used for nested PCR using the HotStarTaq master mix kit (Qiagen, Courtaboeuf, France). The PCR products were purified and directly sequenced using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA). Relevant drug resistance mutations (DRMs) in the protease and RT were identified using the ANRS interpretation algorithms, version 21 (see www.hivfrenchresistance.org/2011/Algo-2011.pdf).

Nucleotide sequences were used to assess the genetic differences between paired sequences obtained from blood plasma samples and DBS by phylogenetic analysis using the MEGA5 software. We constructed phylogenetic trees to identify the HIV-1 subtypes and circulating recombinant forms (CRFs).

Statistical analysis. The results obtained from plasma were considered to be the gold standard. The sensitivity and specificity of the DBS results were calculated in comparison with positive and negative HIV-1 RNA results, respectively, obtained from the plasma samples. Sensitivity was calculated as the ratio of the number of DBS with a VL of ≥1,000 copies/ml to the number of corresponding plasma samples with a VL of ≥1,000 copies/ml. Specificity was calculated as the ratio of the number of DBS with a VL of <1,000 copies/ml to the number of corresponding plasma samples with a VL of <1,000 copies/ml. The ability to detect virological failure (VF) was determined using the VL threshold of 1,000 copies/ml as per WHO recommendations for the surveillance of acquired resistance in developing countries (see http://www.who.int/hiv/pub/drugresistance/drug_resistance_strategy). The correlation between DBS

and plasma HIV-1 RNA levels was assessed with the Spearman rank correlation coefficient for samples with detectable VL. The agreement between the VL results from plasma and DBS was described using Bland-Altman analysis on paired samples with detectable VL. The association between the difference and the mean VL was shown by the coefficient of correlation and tested by the nonzero correlation test. A *t* test was used to determine if the mean difference (bias) was significantly different from 0. The limits of agreement were defined as the mean difference ±1.96 standard deviations (SD).

RESULTS

DBS collection conditions. Overall, we recruited 1,618 patients receiving ART for 24 months, and 1,454 DBS were prepared in parallel with the plasma sample collection. The contribution (number of samples) of each study site was as follows: 262 from lab A, 286 from lab B, 170 from lab C, 293 from lab D, 226 from lab E, and 217 from lab F. Up to 98% of patients had 2 DBS cards collected, each with 5 spots. The ambient temperatures recorded on site during DBS preparation, initial storage, and shipment to the labs varied from 22°C to 30°C, with the lower temperatures being recorded at lab F and the maximum temperature at lab E. Information collected about raining conditions indicated that only 7% of total DBS were collected on rainy days. The duration between DBS preparation and storage at −20°C varied from 16 days to 38 days, with a mean duration of 23 days, and the shipping time varied from a few hours to a maximum of 3 days.

Accuracy of virological failure detection in DBS versus plasma. Three hundred eighty-two plasma-DBS pairs were tested for HIV VL with the VL assays routinely used in each of the national or centrally designated laboratories. Fifty-three to 91 plasma-DBS pairs were tested at each participating site, and at least 20% of the samples tested had a plasma VL of ≥1,000 copies/ml (Table 1). The sensitivities and specificities to detect VF in DBS compared to plasma varied according to the laboratories and detection methods used. Overall, the sensitivities reported by the participating laboratories ranged from 75% to 100% and the specificities from 60% to 98%. Different ranges of sensitivity were also observed according to the techniques used: 75% to 100% for the m2000rt assay in labs B, C, and D, 91% to 94% for the generic G2 assay in labs A and F, and 85% for the NucliSENS assay in lab E. Importantly, the sensitivities varied for the same assay in different laboratories. For example, the sensitivity of the m2000rt assay to detect VF in DBS was 100% in labs B and C but only 75% in lab D. Similarly, the specificity of DBS to detect VF varied also according to the method and the laboratory; the values observed ranged from 83% to 98% for the m2000rt and NucliSENS assays and only around 60% in both laboratories using the generic G2 assay. The specificities differed also for the same method when applied in different laboratories, and ranged, for example, from 83% (lab C) to 98% (labs B and D) for the m2000rt assay. In addition, we observed that the proportion of samples with VL differences of <0.5 $\log_{10}$ copies/ml varied between the different laboratories, ranging between 37% and 92% (Table 1). Overall, of the 155 samples with a plasma VL of ≥1,000 copies/ml, 16 (10.3%) had a DBS VL of <1,000 copies/ml and therefore are missed if the VF threshold using DBS is 1,000 copies/ml. On the other hand, 41 out of the 227 (18.1%) samples with a plasma VL of <1,000 copies/ml had HIV RNA levels in DBS of ≥1,000 copies/ml and may thus represent false VF detection.

Comparison between plasma viral load and DBS viral load. Overall, we found strong positive correlations between DBS VL

TABLE 1 Sensitivities and specificities for detection of virological failure[a] in DBS and plasma

VL assay and lab	Plasma-DBS pairs (n = 382)	No. (%) of pairs with plasma VL of ≥1,000 copies/ml (n = 155)	No. of false negatives/total no. of pairs with plasma VL of ≥1,000 copies/ml[b]	No. of false positives/total no. of pairs with plasma VL of <1,000 copies/ml[c]	Sensitivity (% [95% CI])[d]	Specificity (% [95% CI])	No. of plasma-DBS pairs with detectable VL	VL difference of <0.5 $\log_{10}$ copies/ml (%)
m2000rt assay	173							
Lab B	60	24 (40)	0/24	1/36	100 (85.8–100)	97.2 (85.5–99.9)	26	24 (92)
Lab C	53	13 (25)	0/13	7/40	100 (75.3–100)	82.5 (67.2–92.7)	18	12 (67)
Lab D	60	12 (20)	3/12	1/48	75.0 (42.8–94.5)	97.9 (88.9–99.9)	10	8 (80)
Generic G2 assay	118							
Lab A	60	16 (27)	1/16	17/44	93.8 (69.8–99.8)	61.4 (45.5–75.6)	23	16 (70)
Lab F	58	23 (40)	2/23	14/35	91.3 (72.0–98.9)	60.0 (42.1–76.1)	37	18 (49)
NucliSENS assay	91							
Lab E	91	67 (74)	10/67	1/24	85.1 (74.3–92.6)	95.8 (78.9–99.9)	76	28 (37)

[a] Virological failure is defined as a VL of ≥1,000 copies/ml.
[b] False negatives are samples with a VL of ≥1,000 copies/ml in plasma but a VL of <1,000 copies/ml in DBS.
[c] False positives are samples with a VL of <1,000 copies/ml in plasma but a VL of ≥1,000 copies/ml in DBS.
[d] CI, confidence interval.

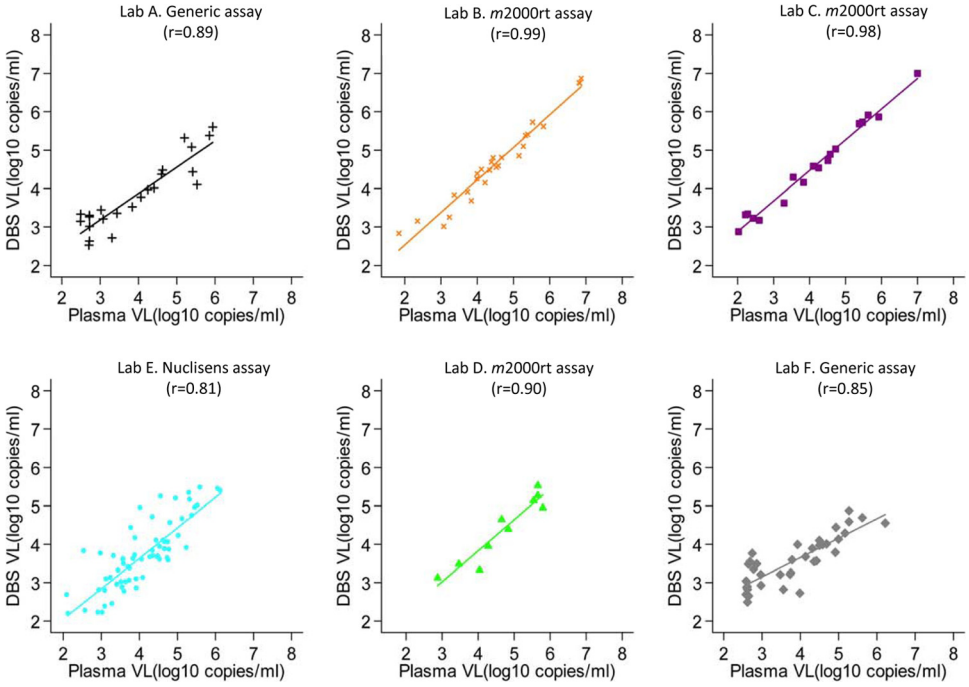

FIG 1 Comparison of viral loads in DBS and corresponding values in plasma only for samples with detectable viral load. Each graphic shows the results of a specific laboratory (lab A to lab F).

and plasma VL in each of the six laboratories, ranging from 0.81 ($P < 0.0001$) to 0.99 ($P < 0.0001$) (Fig. 1). The highest correlations were found for laboratories using the m2000rt method, while the lowest was observed for the NucliSENS assay. Bland-Altman analyses (Fig. 2) indicated a good agreement between the plasma and DBS VL for the NucliSENS assay, but overall, the VL in DBS was underestimated, with a mean difference of −0.44 $\log_{10}$ copies/ml (Fig. 2A). The agreement between the plasma and DBS VL measured with the m2000rt assay was low for a VL of <3,000 copies/ml (3.47 $\log_{10}$ copies/ml) and improved above this threshold (Fig. 2B). For the m2000rt method, the agreement varied also among the laboratories: a good agreement between the DBS and plasma VL was observed for lab B, and a higher and lower VL in DBS compared to that in plasma was seen in labs C and D, respectively. The lowest agreement between the DBS and plasma VL was seen for samples tested with the generic G2 assay, especially when the plasma VL was low (Fig. 2C). For this assay, when only samples with a plasma VL of >3,500 copies/ml were considered, an excellent agreement was observed, but the VL in DBS was still underestimated, with a mean difference of −0.56 $\log_{10}$ copies/ml (Fig. 2D).

HIV drug resistance genotyping using DBS. Using the criteria of a plasma VL of ≥1,000 copies/ml and a DBS VL of ≥5,000 copies/ml to select DBS for genotyping, we tested 78 DBS, of which 67 were PCR positive and 11 were negative after two PCR attempts, for an overall amplification rate of 85.9%. To increase our panel of plasma and DBS paired sequences and thus allow for more robust sequence comparisons between the two materials, we also genotyped DBS with a plasma VL of ≥1,000 copies/ml, regardless of the DBS VL. Using this approach, we selected and genotyped 46 additional DBS, of which 29 (63.0%) were successfully amplified in the *pol* region. The median (interquartile range) plasma VL was 4.1 (3.0 to 5.3) $\log_{10}$ copies/ml for paired DBS samples with successful PCR and 3.7 (3.3 to 4.8) $\log_{10}$ copies/ml for those with negative PCR when using DBS. A total of 96 paired plasma-DBS sequences were then used to compare the sequence homology and drug resistance results. The contributions (number of samples) from each laboratory and the sequence subtype distribution were as follows: 11 from lab A (6 CRF02_AG and 5 CRF06_cpx), 20 from lab B (8 CRF02_AG, 7 CRF36_cpx, 2 CRF22_01A1, 2 G, 1 F2, and 1 unclassified); 11 from lab C (6 CRF02_AG, 2 CRF13_cpx, 1 A1, 1 A3, and 1 CRF49_cpx), 3 from lab D (3 CRF01_AE), 38 from lab E (23 CRF02_AG, 4 A3, 4 G, 2 CRF06_cpx, 1 CRF19_cpx, 1 CRF25_cpx, 1 CRF43_02G, and 2 unclassified), and 13 from lab F (13 CRF01_AE). The amplification rates (DBS VL, ≥5,000 copies/ml) in the laboratories that performed genotyping locally varied from 43% (lab D) to 95% (lab B).

We compared paired plasma and DBS sequences in the viral RT region for overall nucleotide homology. For 22 (22.9%), 61 (63.5%) and 80 (83.3%) samples, we observed <1%, 2%, and 3% nucleotide differences between the plasma and DBS sequences, respectively. This plasma-DBS sequence variation was not uniform across study sites, and interestingly, 8 of the 16 samples with ≥3% nucleotide differences were from one laboratory (lab F).

Drug resistance interpretation in DBS compared to plasma. Seventy-five samples (78.1%) had identical DRM profiles with plasma and DBS. For the 21 (21.9%) samples with differences in their mutation profiles, various interpretations were observed, as summarized in Table 2: two samples had discordant DRM profiles in blood plasma and DBS samples but a similar interpretation for ARV drugs. For 8 samples, viral resistance was detected only in the plasma sequences and no drug resistance mutations were found in

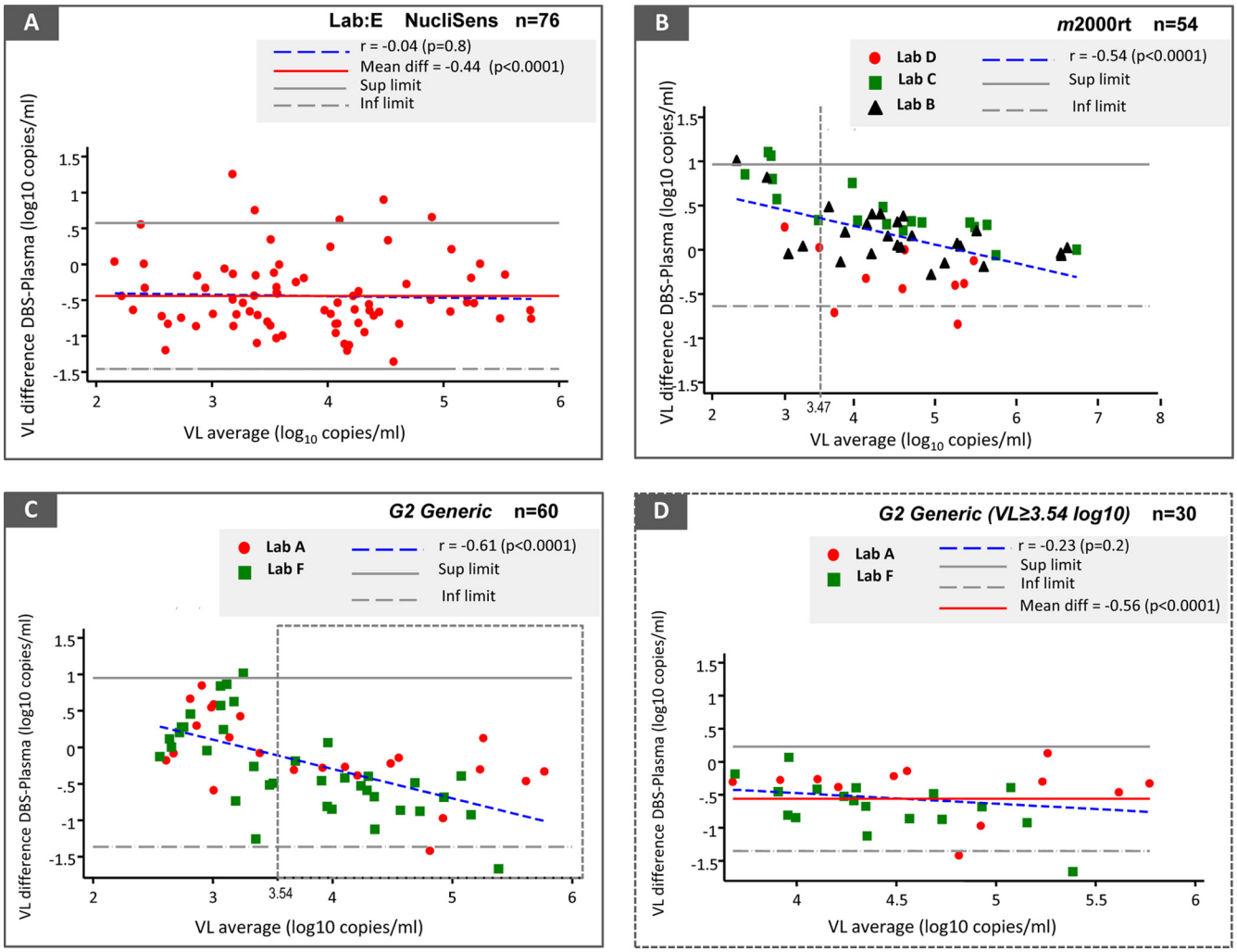

FIG 2 Bland-Altman plots of agreement between viral loads (VL) in paired dried blood spot (DBS) and plasma samples, using different methods: (A) NucliSENS in lab E, (B) m2000rt in labs B, C, and D, and (C) the generic G2 in labs A and F. The dashed line in panel B illustrates the threshold of 3.47 $\log_{10}$ copies/ml above which m2000rt correlation improves. The dashed box in panel C illustrates the better correlation of the generic G2 test of >3.54 $\log_{10}$ copies/ml as represented in panel D.

DBS. More importantly, 6 (75%) of these samples were from the same site (lab F), in which the highest number of samples with ≥3% nucleotide differences was also seen. One sample carried a DRM (K103N mutation) only in the DBS sequence and not in plasma. And finally, 10 samples had different mutation profiles that slightly influenced drug resistance interpretation, leading either to the identification of a reduced number of affected ARVs in DBS ($n = 7$) or the opposite ($n = 3$). The median (interquartile range) DBS VL for the 21 samples with a discordant resistance profile/interpretation was 4.1 (3.6 to 4.6) $\log_{10}$ copies/ml.

DISCUSSION

In resource-limited countries, routine access to HIV VL monitoring and drug resistance assessment to support ART initiation and/or follow-up are still extremely challenging because of the high cost of assays and equipment and limited specialized laboratories and other resources, including trained personnel. Despite these limitations, in the majority of developing countries, at least one national reference laboratory exists that performs VL and/or drug resistance genotyping. Therefore, extending access to VL and genotyping tests in these settings might consist of implementing strategies that allow for the collection of specimens from peripheral and/or remote areas for testing in central laboratories. Dried blood spots might fit in such a strategy, and today, clear guidelines for collection, transport, and storage, including adequate humidity and temperature conditions, have been established for this method (7, 9). In addition, several studies have also indicated that HIV-1 VL quantification and drug resistance genotyping from DBS are feasible and that the results are comparable to those obtained using blood plasma samples (8, 14, 16).

In this study, our main objective was to determine whether or not DBS could reliably replace plasma specimens to identify VF (VL, ≥1,000 copies/ml) and viral resistance in patients failing first-line ART. We conducted this assessment under "real-life" conditions in six sites from low- and middle-income countries to provide results that reflect routine practices. Each laboratory an-

TABLE 2 Drug resistance mutation profiles and interpretation in RT sequences of discordant plasma-DBS genotyping results[a]

Sample ID ($n = 21$)[b]	Mutation profile in:		Drug resistance profile in[c]:		% NT[d] difference in RT	PL VL (log_{10} copies/ml)[e]
	Plasma	DBS	Plasma	DBS		
Different DRM profiles and identical interpretations						
Lab D 2-149	100IL 103N	103N	EFV^r NVP^r	EFV^r NVP^r	0.5	4.7
Lab E 3-341	184V 190A	103S 184V 190A	$3TC^r$ EFV^r FTC^r NVP^r	$3TC^r$ EFV^r FTC^r NVP^r	1.0	4.4
DRMs in plasma sequences only						
Lab F 2-493	103N		EFV^r NVP^r		3.0	5.2
Lab C 2-423	106M 227FL		EFV^r NVP^r		3.0	4.3
Lab E 2-292	98G 106A 184V 227L		$3TC^r$ FTC^r NVP^r		3.9	3.2
Lab F 3-307	103N 184V		$3TC^r$ EFV^r FTC^r NVP^r		3.0	3.8
Lab F 1-622	67DN 90I 181V 184V		$3TC^r$ ETR^r FTC^r RPV^r		3.0	4.5
Lab F 1-701	103KN 181CY 184MV 190A		$3TC^r$ EFV^r FTC^r NVP^r RPV^r		3.7	4.5
Lab F 1-252	101R 179I 181C 184IV 190AG		$3TC^r$ EFV^r ETR^r FTC NVP^r RPV^r		4.0	3.5
Lab F 1-600	65N 75M 90I 181C 184IMV		$3TC^r$ $d4T^r$ EFV^r FTC^r NVP^r RPV^r		2.0	4.0
DRMs in DBS sequences only						
Lab C 4-471		103KN		EFV^r NVP^r	1.0	5.2
Different DRM profiles and interpretations						
Lab B 2-129	103N 184V 221HY	103N 184V	$3TC^r$ EFV^r FTC^r NVP^r RPV^r	$3TC^r$ EFV^r FTC^r NVP^r	0.7	4.0
Lab E 2-055	101Q 179A 181C 184V	101KQ 179A 181C 184V 221HY	$3TC^r$ EFV^r FTC^r NVP^r RPV^r	$3TC^r$ EFV^r ETR^r FTC^r NVP^r RPV^r	1.0	4.1
Lab B 3-175	101HQ 106AV 179I 184V 190A 225HP	101Q 106AV 179I 184V 190A 225HP	$3TC^r$ EFV^r ETR^r FTC^r NVP^r	$3TC^r$ EFV^r FTC^r NVP^r	1.0	5.8
Lab B 1-320	103N 184V 215ST 225H	103N 184V 225H	$3TC^r$ AZT^r $d4T^r$ EFV FTC^r NVP^r	$3TC^r$ EFV^r FTC^r NVP^r	1.0	4.6
Lab E 3-120	103N 138T 179L 181C 184V 221Y	101R 138A	$3TC^r$ EFV^r ETR^r FTC^r NVP^r RPV^r	ETR^r	4.9	3.6
Lab B 3-161	98G 181C 184V 215F	41LM 98G 181C 184V 215F	$3TC^r$ AZT^r $d4T^r$ EFV^r FTC^r NVP^r RPV^r	$3TC^r$ ABC^r AZT^r $d4T^r$ EFV^r FTC^r NVP^r RPV^r	3.0	4.4
Lab E 2-558	179I 181C 184V 215Y	41LM 69ADNT 179I 181C 184V 215Y	$3TC^r$ AZT^r $d4T^r$ EFV^r FTC^r NVP^r RPV^r	$3TC^r$ ABC^r AZT^r $d4T^r$ DDI^r EFV^r FTC^r NVP^r RPV^r TDF^r	1.0	4.4
Lab F 2-451	75M 77L 101KQ 118I 179I 184V 190A 210W 215Y	75M 77L 179I 184V 190A 215Y	$3TC^r$ ABC^r AZT^r $d4T^r$ EFV^r FTC^r NVP^r	$3TC^r$ AZT^r $d4T^r$ EFV^r FTC^r NVP^r	1.0	5.0
Lab A 1-224	41L 103N 108I 184V 210LW 215Y	41L 103N 108I 184V 215Y	$3TC^r$ ABC^r AZT^r $d4T^r$ EFV^r FTC^r NVP^r TDF^r	$3TC^r$ ABC^r AZT^r $d4T^r$ EFV^r FTC^r NVP^r	0.6	5.6
Lab C 1-179	90IV 179I 181CFGV 184V 215FY 221Y	41L 90I 179I 181V 184V 215Y 221Y	$3TC^r$ AZT^r $d4T^r$ EFV^r ETR^r FTC^r NVP^r RPV^r	$3TC^r$ ABC^r AZT^r $d4T^r$ ETR^r FTC^r RPV^r	2.0	4.1

[a] RT, reverse transcriptase; DBS, dried blood spot.
[b] ID, identification; DRMs, drug resistance mutations.
[c] Interpretation algorithm used was the Agence Nationale de Recherches sur le Sida et les Hépatites Virales (ANRS) version 21. 3TC, lamivudine, d4T, stavudine, AZT, zidovudine, NVP, nevirapine, EFV, efavirenz; FTC, emtricitabine; ABC, abacavir; DDI, didanosine; TDF, tenofovir; ETR, etravirine; RPV, rilpivirine.
[d] NT, nucleotide.
[e] PL, plasma.

alyzed DBS samples according to the routine procedures used for VL and/or drug resistance, for which they adapted as much as possible the constraints and limitations of DBS to their available equipment and programmatic context. Our results showed that using DBS to detect VF and drug resistance is feasible in RLC, either in African or in Asian countries. Although the work was conducted in different settings/laboratories, the overall results showed that 90% (139/155) of VF were correctly identified using DBS, and drug resistance interpretation in DBS was correct for 80% (77/96) of samples. This positive outcome of the DBS strategy has already been reported, but generally in a single setting or with less diverse platforms or methods (16–18), and it indicates that DBS is an interesting option for national programs. However, our results also identified some limitations of this approach that necessitate specific attention in order to improve the DBS strategy.

Although the three viral load methods used in this study have been reported to perform well when using plasma samples and to detect a broad range of HIV-1 non-B strains (19, 20) with lower quantification limits ranging from 40 copies/ml for the Abbott m2000rt assay to 300 copies/ml for the generic G2 assay, they displayed variable performance when using DBS. The NucliSENS EasyQ assay, which measures RNA only, showed an overall good agreement between the plasma and DBS VL. However, this assay underquantified HIV-1 RNA in DBS, with a mean difference of -0.44 $\log_{10}$ copies/ml, which is similar to the findings of other reports (21). Because of this underestimation, the assay showed the lower sensitivity to detect VF. This underquantification can be associated with the degradation of RNA on DBS. Our results are based on data from a single laboratory, but similar findings have been reported in other studies (22).

The Abbott m2000rt assay, which the manufacturer claims preferentially extracts RNA, showed the highest sensitivity in at least two of the three laboratories that use the assay. However, an overquantification of the VL in DBS was observed in pairs with a plasma VL of <3,000 copies/ml, likely due to proviral DNA and intracellular RNA contaminations. Similar observations were recently made by Vidya and colleagues (23) and they reported the same threshold of 3,000 copies/ml. Other reports have also mentioned the reduced performance of the m2000rt assay on DBS for a plasma VL of <3,000 copies/ml (18, 24). The generic G2 assay developed by the ANRS Working Group on HIV Quantification is used in many laboratories in developing countries because of its lower cost than the costs of commercial assays. This assay has been shown to perform equally as well as commercial tests on plasma and detects and quantifies a wide diversity of HIV-1 group M subtypes and CRFs (19, 25). The concordant results observed in the two laboratories from our study that used this assay indicate a relatively good sensitivity but a very poor specificity, especially for samples with a low plasma VL (<3,500 copies/ml). That observation, already made at a lower scale for the m2000rt method, was amplified for the generic G2 assay and indicates that this assay hardly discriminates between proviral DNA and circulating RNA, as we already reported (9, 11). Indeed, when a higher cutoff (plasma VL ≥ 3,500 copies/ml) was used to eliminate the impact of proviral DNA, the specificity increased but the sensitivity dropped significantly, indicating that this assay underquantified VL in DBS as observed for the NucliSENS assay. Solutions to overcome the impact of proviral DNA include the consideration of a higher cutoff for VF (e.g., ≥5,000 copies/ml), as per WHO recommendations for public health in developing countries (26), but that limits the assessments of VF and drug resistance in patients who can be identified between 1,000 and 5,000 copies/ml; alternatively, a DNase treatment can be considered for DBS, as was already suggested (27), but the costs/benefits of this additional step, including the operational aspects and ease of processing when testing large sample series, should be clearly assessed.

For drug resistance genotyping on DBS, we showed that below the threshold of 5,000 copies/ml (plasma VL), the amplification rate drops significantly and thus many PCR attempts are required, most likely because of nucleic acid degradation; there is a substantial cost impact involved because of the repeated PCRs. In addition, it is possible that some sequences obtained from DBS, especially for samples with low VL, may not reflect the circulating virus and thus can affect resistance interpretation. This limitation of drug resistance genotyping using DBS is well known (7, 11), and no consensus solution currently exists. These limitations of DBS for drug resistance genotyping may explain the results observed in lab F, and we also cannot exclude performance issues for PCR testing and sequence editing in this lab. We believe, however, that in areas where plasma collection and processing are not feasible to provide critical responses to patients or national programs, the benefit of using DBS should be clearly considered.

Overall, our study shows that DBS can be used as an alternative to plasma for VL and genotypic drug resistance testing, especially in areas with limited infrastructures. However, our results showed that all VL assays do not perform equally on DBS, and if applicable, this aspect should be considered, e.g., when implementing new laboratories in the country. Program and/or laboratory managers should conduct in-country evaluations to ensure that the test/method is best adapted to their local conditions (available facilities, viral diversity, operational characteristics, price, etc.). More importantly, our study, performed under field conditions, revealed interlaboratory differences independent of the methods used for VL or genotypic drug resistance testing. These observations highlight the need for the regular training of laboratory personnel performing these tests, not only for DBS, since similar issues are reported for plasma in various settings (28). In addition, participation in national and/or international proficiency testing programs should be mandatory for laboratories performing VL and drug resistance genotyping on DBS and/or blood plasma samples.

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